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Deletion of Stat3 Blocks Mammary Gland Involution and Extends Functional Competence of the Secretory Epithelium in the Absence of Lactogenic Stimuli

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The transcription factor Stat3 is activated through tyrosine phosphorylation by many cytokines and is a fundamental mediator of their signals. In the mammary gland, Stat3 activity increases sharply shortly after weaning, and involution is delayed in mice, that contain a mutant Stat3 lacking 33 amino acids including the key tyrosine residue. We have now generated a more extensive mutation of Stat3 through the deletion of exons 15–21 in mammary epithelium. This resulted in the loss of 245 amino acids including the DNA binding and SH2 domains, and Stat3 protein was undetectable. Pregnancy-mediated mammary development and lactation were normal in these mice. Involution was delayed and, remarkably, Stat3-null mammary epithelium maintained its functional integrity and competence even 6 d after weaning, whereas control mammary tissue was rendered nonfunctional within 2 d. The lack of remodeling and functional stasis of the epithelium correlated with the disruption of proteinase activity. Our data demonstrate that mammary tissue can retain its functional competence in the absence of external lactogenic stimuli and demonstrate a delay in the initiation of the irreversible stage of involution. (*Endocrinology* 143: 3641–3650, 2002)

EPITHELIAL CELL DEATH and remodeling of mammary tissue occur during involution and are regulated by changes in systemic hormones and local growth factors as well as interactions between the epithelium and the extracellular matrix (1, 2). The initiation of involution is characterized by changes in the activity of two members of the signal transducer and activators of transcription (STAT) family of transcription factors. The phosphorylation status, and thereby their activity, of the transcription factors Stat5 and Stat3 changes rapidly at the onset of involution (3). Stat5a and Stat3 have reciprocal patterns of phosphorylation with levels of Stat5a decreasing and Stat3 increasing at the beginning of involution. These changes can be detected within 12 h after weaning of the pups (3, 4). In the mammary gland, Stat5 phosphorylation and activation can be induced by prolactin (PRL), epidermal growth factor (EGF), and GH (5) whereas the factors that activate Stat3 during involution are unknown, but possible candidates include members of the IL-6 cytokine family, EGF, platelet-derived growth factor (PDGF), and IGF pathways (6–8).

The inactivation of Stat5 and Stat3 has demonstrated unique roles for these transcription factors in mammary physiology. Inactivation of the genes encoding Stat5a and 5b has revealed their essential role in the proliferation and differentiation of mammary alveolar epithelium during pregnancy (9, 10). In addition, Stat5a is a survival factor for mammary epithelium in the presence of a proliferative growth stimulus (11). Because inactivation of the Stat3 gene results in embryonic lethality, the Cre-loxP recombination system had been used to inactivate this gene in mammary epithelium. The deletion of exon 22, which encodes a critical tyrosine residue, demonstrated a role for Stat3 in the regulation of the first phase of involution (12). Apoptosis of mammary epithelial cells was reduced during the first phase, but involution was able to proceed after a delay of 3 d. This result implicates that Stat3 may not play the dominant role in the regulation of all stages of involution and that additional factors control cell death and tissue remodeling. Alternatively, it is possible that this mutant Stat3, which lacked only 33 amino acids, retained some activity. In T cells, a mutant Stat3 with a loss of the region encoding the tyrosine residue at 705 possessed a dominant negative function (13).

In an attempt to further clarify and define the molecular roles of Stat3 in the process of mammary tissue remodeling, we completely inactivated the Stat3 gene in mammary epithelium using a whey acidic protein (WAP)-Cre transgene (WC) to excise exons 15–21 that had been bracketed by loxP sites (14). This region encodes 245 amino acids including the DNA binding and SH2 domains. These experiments permitted us to address whether functional competence of mammary tissue upon weaning can be maintained in the absence of Stat3, and whether the protease-mediated second stage of involution depends on the presence of Stat3.

Abbreviations: EGF, Epidermal growth factor; MMP, matrix metalloprotease; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3-kinase; PRL, prolactin; SG-P-2, sulfated glycoprotein 2; STAT, signal transducer and activators of transcription; TBST, Tris-buffered saline with Tween 20; TUNEL, terminal deoxyuridine triphosphate nick end labeling; WAP, whey acidic protein; WC, WAP-Cre transgene.

Materials and Methods

Materials

The generation of Stat5a antibodies has been described (3). Primary antibodies for antiphosphotyrosine and phosphatidylinositol 3-kinase

(PI3K) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Primary antibodies for β-catenin, actin, STAT3 (C-20), Bcl-x (S-18), Bax (N-20), STAT1 (E-23), and SGP-2 (M-18) were purchased from Santa Cruz Biotechnology, Inc. (La Jolla, CA). Goat antirabbit and rabbit antimouse secondary antibodies were purchased from Transduction Laboratories, Inc. (Lexington, KY). Biotin-labeled deoxyuridine triphosphate and terminal transferase were purchased from Roche Molecular Biochemicals (Indianapolis, IN). Super Signal Western Detection Kit was purchased from Pierce Chemical Co. (Rockford, IL).

Generation of STAT 3 fl/fl;WC

Creation of the mice that carry two Stat3 floxed alleles (Stat3 fl/fl) mice has been described previously (14). Briefly, a conditional allele of Stat3 was generated by homologous recombination with loxP sites inserted adjacent to exons 15 and 21 of the mouse Stat3 gene. A single male Stat3 fl/fl (SV129/C57BL6) founder was interbred with WAP-Cre (FvB/C57BL6) transgenic female mice to generate F1 founders heterozygous for the Stat3 floxed allele (Stat3 fl/+) and transgenic for WAP-Cre (Stat3 fl/+;WC). The resulting offspring were backcrossed five times to generate mice homozygous for Stat3 flox and transgenic for WAP-Cre. The same generation nontransgenic Stat3 fl/fl and nontransgenic Stat3 fl/+ littermates (Stat3) were used as controls for all molecular analyses. PCR confirmation of the presence of the Cre transgene and the Stat3 flox and null alleles has been described (14, 15). Genotype of the mice was confirmed after tissue collection by Western analysis for Stat3 expression. All animals were housed and handled according to the approved protocol established by the Institutional Animal Care and Use Committee and NIH guidelines.

Mammary gland collection

Mammary glands were surgically removed from anesthetized (and cervically dislocated if no further time points were needed) mice at d 10 of lactation and d 2, 6, and 9 of involution. Biopsies were performed on anesthetized (isoflurane gas) dams at the desired time point in a survival surgery conducted according to the approved protocol established by the Institutional Animal Care and Use Committee and NIH guidelines. The biopsies have not been found to alter the progression of involution in this or any prior studies using the surgical excision. Day 1 of involution was designated as 24 h after the morning that the pups were removed from the dam. Dams were allowed to lactate for 10 d, and then the pups were removed to induce involution. The mammary lymph node was removed before homogenization of all glands. Tissues were prepared immediately for RNA and protein extraction as described. Inguinal (number 4) mammary glands used for histological analysis were surgically excised, spread on Omniset tissue cages (Fisher Scientific, Pittsburgh, PA), fixed for 5 h in Tellyzinsky's fixative or overnight at 4 C in 10% neutral buffer formalin. Glands were stored in 70% ethanol until processed by standard embedding and sectioning techniques onto Probe-On Plus slides (Fisher Scientific). Sections were stained with standard hemotoxylin and eosin.

Western analysis

Preparation of protein extracts for Western analysis has been described (3, 9, 11). Briefly, 2 mg of fresh and frozen tissue were homogenized in 2 ml of lysis buffer with protease inhibitors; phenylmethylsulfonyl fluoride, leupeptin, and aprotinin at 50 μg/ml and the activated phosphotyrosyl-proteinase inhibitor sodium orthovanadate at 4 mM on ice. Protein lysates were rocked for 1 h at 4 C and then cleared by centrifugation at 14,000 × *g* for 20 min. Protein concentration in the lysates was determined using Bio-Rad Laboratories, Inc. (Hercules, CA) Protein Assay Kit according to the manufacturer's protocol. Lysates containing 40 μg of protein were mixed with 2× loading buffer and 2-mercaptoethanol then heated to 90 C for 3 min. Samples were briefly spun, electrophoresed, under denaturing conditions, on 8%, 12%, or 14% precast Tris-glycine gels and transferred to polyvinylidendifluoride membranes according to the manufacturer's protocol (Novex, San Diego, CA). Western analysis was performed essentially as described with the following exceptions; primary antibody (dilution Stat5a 1:5000; Stat3 1:1000; Bax 1:1000; antiphosphotyrosine 1: 5000; Bcl-x 1:1000, PI3K 1:1000, WAP 1:5000, β-catenin 1:500, actin 1:5000) was incubated overnight at 4 C with gentle rocking and all incubations with antibodies and initial blocking was performed in 3% nonfat dried milk in 1× TBST (Tris-buffered saline with Tween 20). Detection was performed with the Super Signal Western Detection Kit according to manufacturer's protocol (Pierce Chemical Co.) and exposed to Kodak MR autoradiography film (Rochester, NY). Exposure times were between 1 sec and 2 min. Immunoprecipitations with anti-Stat5a and anti-Stat3 antibodies were carried out essentially as previously described (11). Stripping was performed by incubating blots for 25 min at room temperature in Antibody Stripping Solution from the Western Blot Recycling Kit according to the manufacturer's protocol (Alpha Diagnostic, San Antonio, TX). Blots were washed in TBST and then blocked with 3% nonfat dried milk in TBST. Membranes were stripped and reprobed up to four successive times. A loading control gel was generated from a parallel SDS-PAGE gel that was fixed for 2 h in 50% methanol, 10% acetic acid, stained overnight in 50% methanol, 0.05% Brilliant Cresyl Blue. Gel was destained in 5% methanol, 7% acetic acid until the background cleared. Gel was dried according to the manufacturer's protocol (Research Products International, Mount Prospect, IL). The Western data shown are representative of separate analyses that have been performed in triplicate using different sample sets (12 Stat3-null and 6 control mice). Stat3 and Stat5a analyses have been performed on every blot to ensure deletion and compare the relative loading between blots.

Immunohistochemistry of STAT3 and STAT5a

Immunohistochemical detection of Stat3 and Stat5a has been described previously. Briefly, 4% neutral buffered formalin fixed, 5-μm tissue sections were deparaffinized, incubated in tissue unmasking fluid (Vector Laboratories, Inc., Burlingame, CA), and heated in a microwave for 2 min. Sections were allowed to cool for 10 min and then washed in PBS, blocked with 10% normal goat serum for 30 min at room temperature and then incubated overnight at 4 C with primary antibody at a dilution of 1:200 for Stat3 and 1:600 for Stat5a. A standard secondary antibody detection protocol with biotin avidin HRP antibodies identified the presence of antigen. Sections were counterstained with hemotoxylin and dehydrated according to standard protocols.

Zymogram gel analysis

Coomassie stained gelatin zymogram SDS-PAGE was carried out using 40 μg of protein from whole gland lysate essentially as described in the manufacturer's protocol (Novex/Invitrogen). Gelatin zymography was performed using unstained 10% gelatin embedded Tris-glycine gels from Invitrogen. After electrophoresis, renaturing, and developing the gelatin embedded Tris-glycine SDS-PAGE gels were fixed for 2 h in 50% methanol, 10% acetic acid, stained overnight in 50% methanol, 0.05% Brilliant Cresyl Blue. Gel was destained in 5% methanol, 7% acetic acid until the background cleared. Gel was dried according to the manufacturer's protocol (Research Products International, Mount Prospect, IL). Preparation and quantitation of protein lysates were described in the previous section describing the Western analysis. The image was captured in grayscale to demonstrate a clear contrast in loading and protease activity.

TUNEL assays

Protocols for TUNEL (terminal deoxyuridine triphosphate nick end labeling) analysis have been previously described (11). Tissue sections from mammary glands analyzed for Stat3 deletion by Western analysis were used for the TUNEL analysis. Each apoptosis sample counted represents a minimum of three random fields (at ×200 magnification) and a minimum of 1000 total cells per tissue section for each mouse. A minimum of three mice per time point for each genotype were collected and analyzed. We were able to use a Z-distribution for the statistical analysis of TUNEL positive cells with the data sets that included three mice each contributing 1000 individual units (cells counted), and a total of 3000 cells counted for each time point and genotype.

Results

Inactivation of the Stat3 gene in mammary epithelium

Stat3 fl/fl mice (14) were bred with WC mice (15) to generate Stat3 fl/fl;WC dams, which were subsequently mated.

The WAP-Cre transgene is highly active during pregnancy, and Cre-mediated recombination can be observed in the majority of mammary epithelial cells (16). The extent of excision of exons 15–21 and the loss of Stat3 protein were investigated by Southern and Western blot analyses, respectively. Genomic DNA was prepared using mammary tissue from Stat3 fl/+;WC and control mice at d 18 of pregnancy and d 8 of involution. The genomic DNA was analyzed for the presence of the wild-type, recombined and floxed alleles (Fig. 1A). At d 18 of pregnancy the recombined allele was predominant, suggesting efficient deletion of exons 15–21 of the *Stat3* gene. The presence of an unrecombined allele is indicative of the presence of stroma, where the WAP-Cre gene is not active and the presence of the wild-type allele.

To verify the absence of Stat3 upon inactivation of the *Stat3* gene we analyzed Stat3 on Western blots at d 10 of lactation, and d 2 and 6 after weaning (Fig. 1B). No Stat3 was detected in mammary tissue from Stat3 fl/fl;WC mice. In contrast Stat3 was detected in mammary tissue from wild-type mice at d 10 of lactation and increased levels at d 2 of involution (Fig. 1B). Stat3 is preferentially expressed in mammary epithelium and the stroma contributes little to the signal seen in normal mammary tissue. We also examined the expression of Stat1 and Stat5 that could potentially compensate for the absence of Stat3 (Fig. 1B). Stat5 levels decreased after weaning and Stat1 levels increased. The expression patterns of these STATs were similar in both Stat3 fl/fl;WC and control mice.

Expression of WAP and sulfated glycoprotein 2 (SGP-2) are maintained in Stat3-null mammary epithelium during involution

We examined the presence of milk proteins and other proteins linked to the maintenance and regression of mammary tissue. The milk protein WAP was detected in Stat3-null mammary tissue at d 10 of lactation followed by an increase at d 2 of involution (Fig. 1C). In contrast, WAP was not detected at d 2 of involution in wild-type controls. SGP-2 was detected during lactation in Stat3 fl/fl tissues, and its level was increased at d 2 of involution followed by a decrease at d 6 of involution (Fig. 1C). In contrast, SGP-2 levels remained high at d 6 of involution in Stat3fl/fl;WC mammary tissue (Fig. 1C). We also examined the expression patterns of proteins linked to cell survival, cell death and cell integrity. The expression patterns of Bcl-x, Bax, and PI3K were comparable between control and Stat3-null mammary tissue (Fig. 1D).

Loss of Stat3 prolongs mammary involution

Stat3 fl/fl;WC and Stat3 fl/fl mice were mated and at d 10 of lactation the pups were removed. The integrity of mammary tissue was analyzed at d 10 of lactation and d 2, 6, and 9 of involution (Fig. 2). There was lack of epithelial remodeling in Stat3-null mammary tissue during these stages and an apparent failure to cease secretion at a time that corresponded to the second, irreversible stage of involution. The presence of lipid droplets and eosinophilic material within

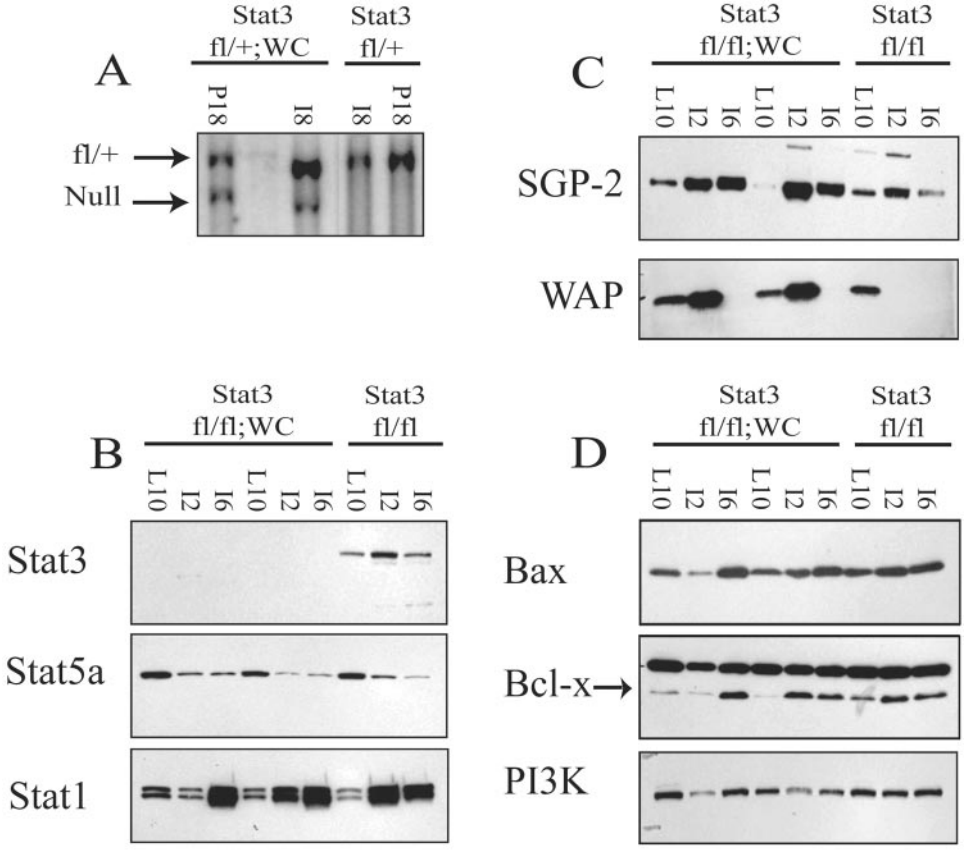

FIG. 1. Southern and Western blots demonstrating recombination of the Stat3 gene and Stat3 protein levels. DNA was analyzed from mammary tissue from d 18 of pregnancy and d 8 of involution from Stat3 fl/+;WC and Stat3 fl/+ mice (A). The floxed (fl/+) and recombined (null) alleles are shown. P18, pregnancy d 18; I8, involution d 8. The time points of P18 and I8 were selected to demonstrate the excision of the floxed exons before and after the points used for analysis. Proteins were analyzed at d 10 of lactation (L10) and d 2 (I2), and 6 (I6) of involution. A, Southern blot demonstrating recombination in fl/+ mice expressing WAP-Cre and fl/+ controls that do not express Cre. Separate mice were used for each time point with a total of four mice used for the Southern analysis. B, Western blot demonstrating the loss of Stat3 in mammary tissue from Stat3 fl/fl;WC mice. Stat5a levels decrease during involution in the absence and presence of Stat3. Stat1 is induced during involution in the absence and presence of Stat3. C, Western blot demonstrating differential expression of SGP-2 and WAP. D, Western blots showing the levels of Bax, Bcl-x, and PI3K.

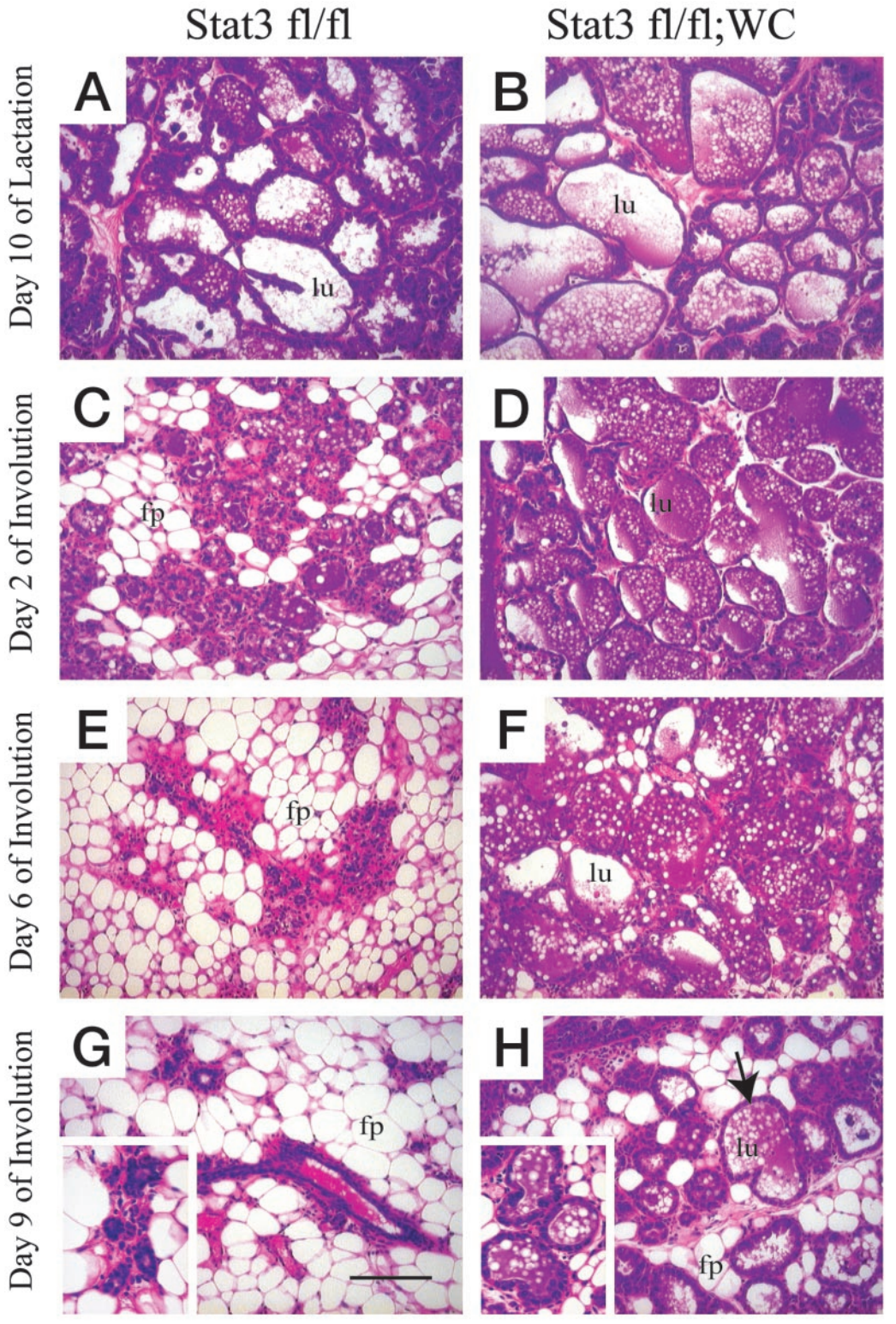

FIG. 2. Delayed involution of mammary epithelium in the absence of Stat3. Mammary tissue from Stat3 fl/fl (A, C, E, G) and Stat3 fl/fl;WC mice (B, D, F, H). At d 10 of lactation, there is very little difference between the Stat3 fl/fl and Stat3 fl/fl;WC tissues (A, B). The fat pad is filled and the alveoli formed lumina with lipid droplets. At d 2 of involution, the differences between Stat3 fl/fl and Stat3fl/fl;WC tissues are visible (C, D). The fat cells reappeared with the collapse of alveoli in Stat3 fl/fl tissue whereas the Stat3 fl/fl;WC tissue retained its integrity. At d 6 of involution, Stat3 fl/fl mammary tissue has initiated extensive remodeling (E), whereas Stat3 fl/fl;WC tissue retained secretory alveoli that demonstrate a delay in the initiation of the second stage of involution (F). At d 9 of involution, Stat3 fl/fl tissue was similar to the virgin state, whereas secretory structures were retained in Stat3 fl/fl;WC tissue. Lipid droplets and expanded lumina were observed at d 9 of involution. Each *inset* is at ×2 relative magnification to further illustrate the collapse of the Stat3 fl/fl lumen, whereas the Stat3 fl/fl;WC lumina remains expanded. The *solid bar* represents 200 μm.

the alveoli at d 6 (Fig. 2F) suggested that the majority of alveolar structures had retained a functional lactogenic phenotype. We did not observe any significant epithelial regression until d 14 after the initiation of involution (data not shown). The regression at d 14 of involution in the Stat3 null gland resembled the control samples at d 6 of involution. After 45 d of involution, the mutant glands were completely remodeled. Dams had no apparent difficulty nursing their pups during the second litter, and no significant difference was noted when the pregnancy occurred during the postpartum estrous. However, after their third pregnancy dams often displayed symptoms of mastitis and invasion of the mammary tissues by lymphocytes unless they had been allowed to involute for at least 3 wk before the third pregnancy. The symptoms of apparent infection and mastitis are similar to results observed in the previous Stat3 study (12).

Lactogenic competence is maintained upon weaning in the absence of Stat3

We hypothesized that, based on the apparent structural integrity, Stat3-null mammary tissue may have failed to enter the second irreversible phase of involution, and thus might be capable of reinitiating lactogenic function. To test this hypothesis, we investigated the functional state of Stat3-null glands after weaning. We allowed Stat3 fl/fl;WC and Stat3 fl/fl control mice to reach d 10 of lactation and removed the pups. After 6 d of involution, one no. 4 gland was surgically removed and 5-d-old pups were placed onto these dams. The pups were then allowed to suckle for 5 d. Ninety percent of the pups (18 of 20) placed onto the Stat3 fl/fl;WC dams survived. In contrast, all of the 12 pups placed onto the Stat3 fl/fl control dams died on or before 5 d of suckling. These results demonstrated that the Stat3-null mammary glands were held in a functional stasis despite the loss of external lactogenic stimuli, and that lactation could resume after 6 d of involution. Histological analyses confirmed that Stat3-null mammary tissue displayed functional alveolar structures 5 d after the reinitiation of lactation, whereas control tissue did not display any secretory alveolar structures (Fig. 3).

Activation of Stat5 and WAP expression upon reinitiation of lactation in Stat3-null glands

A hallmark of lactation is the PRL-mediated activation and nuclear translocation of Stat5 in mammary epithelium. To establish whether Stat5 was activated in Stat3-null mammary epithelium that had gone through 6 d of involution followed by 5 d of suckling, we performed immunohistochemical analyses. Nuclear Stat5a was detected in the Stat3-null mam-

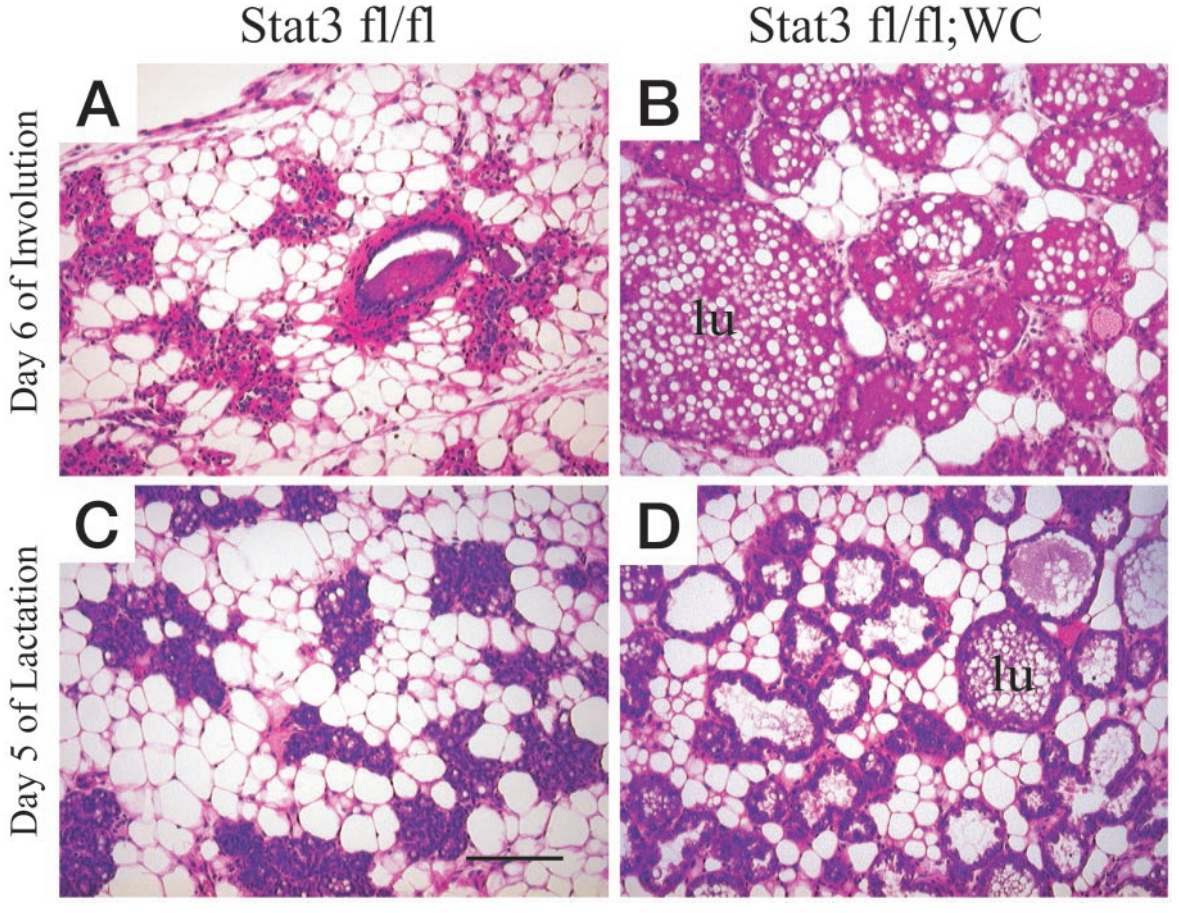

FIG. 3. Reestablishment of lactation in the absence of Stat3 after 6 d of involution. Mammary tissue from Stat3 fl/fl (A, C) and Stat3 fl/fl;WC mice (B, D). At d 6 of involution, Stat3 fl/fl mammary epithelium had been extensively remodeled, whereas Stat3 fl/fl;WC tissue had retained secretory features. Pups were placed with these dams, and after 5 d of suckling secretory features were seen in Stat3 fl/fl;WC but not Stat3 fl/fl mice. Stat3 fl/fl tissue did not respond to the external suckling stimulus. The *solid bar* represents 200 μm.

mary epithelium after 6 d of involution followed by 5 d of lactation (Fig. 4A, panel d). Western blot analysis of these glands revealed that WAP levels had also increased after 5 d of suckling (Fig. 4C). These data demonstrate that the Stat3-null epithelium could reinitiate lactation after 6 d of forced involution, and display appropriate physiological markers.

The absence of Stat3 in the mammary epithelium of Stat3 fl/fl;WC mice was confirmed by immunohistochemical analysis of sections from d 6 of involution and after 5 d of suckling. No nuclear Stat3 was observed in Stat3 fl/fl;WC mammary epithelium demonstrating that the gene had undergone Cre-mediated recombination (Fig. 4B, panels b and d). In contrast nuclear Stat3 was observed in Stat3 fl/fl mammary epithelium (Fig. 4B, panels a and c).

Reduced levels of apoptosis in the absence of Stat3

One of the hallmarks of mammary gland involution is the increase in programmed cell death and an increase in the expression of genes involved in the regulation of programmed cell death (2). We examined the level of apoptosis in mammary tissue from Stat3 fl/fl;WC and Stat3 fl/fl control mice by TUNEL analysis (Fig. 5). The percentages of TUNEL positive cells were determined for Stat3 fl/fl;WC and Stat3 fl/fl tissues at 10 d of lactation and d 2 and d 6 of involution. The percentages obtained were 0.14 (± 0.03), 0.86 (± 0.03), and 1.16 (± 0.11) for Stat3 fl/fl;WC at 10 d of lactation, d 2 and d 6 of involution, respectively. The percentages obtained for the Stat3 fl/fl control tissues were 0.20 (± 0.10), 2.73 (± 0.19), and 2.52 (± 0.71) for 10 d of lactation, d 2 and d 6 of involution, respectively. The level of apoptosis was significantly reduced ($P < 0.05$) at d 2 of involution (significance determined using a Z-distribution, n=3 mice with 1000 cells counted per mouse, and a total of 3000 cells counted at each time point for both Stat3 fl/fl and Stat3 fl/fl;WC using random fields for analysis). At 6 d of involution no significant difference could be determined within an appropriate statistical confidence interval between the Stat3 fl/fl and Stat3 fl/fl;WC tissues. Levels of TUNEL positive cells at d 2 or 6 of involution in the Stat3 null tissues were very low but not entirely absent. We conclude from this data that the program that initiates programmed cell death in the mammary gland is debilitated in the absence of Stat3.

Altered protease activity in the absence of Stat3

A hallmark of involution of the mammary gland is the change in expression of proteases controlling the degradation of extracellular matrix, in particular the matrix metalloproteases (MMPs) and their inhibitors (17, 18). Because the loss of Stat3 prevented mammary tissue from being remodeled, we hypothesized a delay in the activation of extracellular MMP activity. To test this hypothesis, we investigated the pattern of protease activity using SDS-PAGE zymogram assays. Protein lysates from Stat3 fl/fl;WC and Stat3 fl/fl mammary tissue were separated in unstained gelatin embedded polyacrylamide gels, and the presence of protease activity was evaluated by the appearance of clear bands after Coomassie staining. We detected several differences in the pattern of protease activities between Stat3-null and control mammary tissue (Fig. 6). MMP9 activity (92k) in Stat3-null samples during involution was delayed (Fig. 6, *arrow 2*). In contrast, bands corresponding to the 72k proform and 62k active MMP2 did not show significant difference between the Stat3-null and control samples (Fig. 6, *arrows 3 and 4*, respectively).

Discussion

We have deleted exons 15–21 of the *Stat3* gene in mammary epithelium during pregnancy using a WAP-Cre transgene that reliably recombined loxP sites located in different genes during pregnancy (15, 16, 19). The deleted sequence of 245 amino acids includes the DNA binding and SH2 domains. Although the excision generated an in-frame deletion, no truncated protein was detected, suggesting that it is unstable. The loss of Stat3 had profound effects on the progression of involution, both morphologically and functionally. There was little loss of epithelial cells and little remodeling in the first 6 d of involution, and even after 9 d secretory structures were evident. In contrast, remodeling was observed in the presence of Stat3 within 2 d after weaning. Notably, the Stat3 null mammary gland retained its structural integrity and functional competence even after 6 d of involution in the absence of external lactogenic stimuli. Importantly, lactation was reestablished in Stat3-null mammary epithelium after 6 d of involution, which was associated with an increase of nuclear Stat5a and WAP, hallmarks of active signaling through the PRL receptor. This argues that the effects of the loss of Stat3 on the mammary epithelium are dominant over changes that occur in response to systemic hormonal changes. It could be argued that the loss of Stat3 in cell types other than the mammary epithelium contributes to the delayed involution, possibly by changing the hormonal balance. However, this is unlikely as the WAP-Cre transgene has been used to delete several genes and tight mammary-specific recombination was observed in all cases. To completely rule out indirect influences, it was necessary to perform mammary transplantation experiments. We have transplanted mammary epithelium from Stat3 fl/fl; WC and Stat3 fl/fl; MMTV-Cre mice into wild-type hosts and investigated pregnancy-induced mammary development, which appeared to be normal in both cases (our unpublished data). Because transplanted tissue does not have a connection to the nipple, it undergoes rapid remodeling after parturition and involution studies could not be performed at the time points needed for a direct comparison.

Our study confirms and extends previous research on the role of Stat3 in mammary gland involution (12). Although both studies point to a critical role of Stat3 in the process of involution, there are distinct differences between the observed phenotypes. We demonstrate that mammary tissue retained functional competence, whereas in the previous study the deletion of 33 amino acids resulted in a delay of involution and no functional rescue was reported. The differences could be the result of the targeting strategy and the level of Cre-mediated excision of Stat3. Although the previous study generated a Stat3 that left the DNA binding and SH2 domains intact, no Stat3 protein was detected in our study. The truncated Stat3 protein used by Chapman *et al.* (12) has been associated with a dominant negative effect in

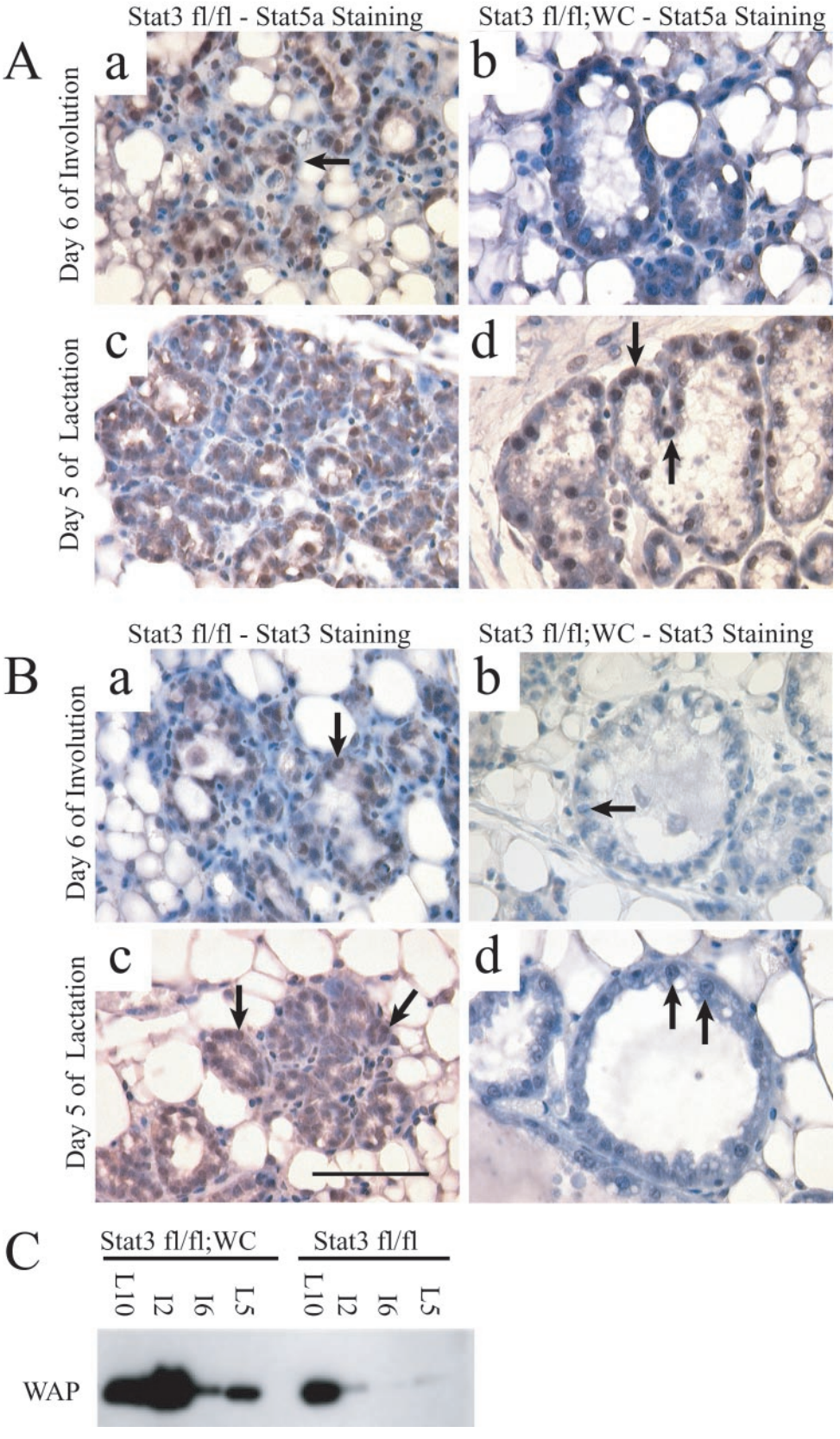

FIG. 4. Stat5 and Stat3 activity after reestablishment of lactation. Dams were allowed to lactate for 10 d then the pups were removed to initiate involution. A, Stat5a immunohistochemistry in tissues from d 6 of involution (a, b) and d 5 of suckling after 6 d of involution (c, d). a and c, Stat3 fl/fl tissue showing cytoplasmic and nuclear Stat5a at d 6 involution and a more dominant cytoplasmic staining at d 5 of suckling. b and d, Stat3 fl/fl;WC tissue with an abundance of cytoplasmic Stat5a at d 6 of involution and an increase in nuclear Stat5a at d 5 of reestablished lactation. *Arrows* indicate nuclei. B, Stat3 immunohistochemistry in tissues from d 6 of involution (a, b) and d 5 of suckling after 6 d of involution (c, d). a and c, Stat3 fl/fl tissues demonstrating the presence of cytoplasmic and nuclear Stat3 protein. b and d, Stat3 fl/fl;WC tissue; Stat3 protein is below a detectable level in the cytoplasm and nucleus of the alveolar epithelium indicating the loss of Stat3. *Arrows* indicate nuclei. C, Western blot showing the reinduction of WAP in Stat3 fl/fl;WC tissue at d 5 of suckling after 6 d of involution. The tissues used for lactation d 10 were biopsied from the same donors used for the d 2 involution time points. The tissues used for d 6 of involution were biopsied from the same donors used for the reinitiated lactation time points. Biopsies were conducted by surgically removing one no. 4 gland for the initial time point and the contralateral gland was collected after the specified period of involution. Induction of WAP was observed in the Stat3 fl/fl;WC tissue. *Solid bar* represents 125 μm.

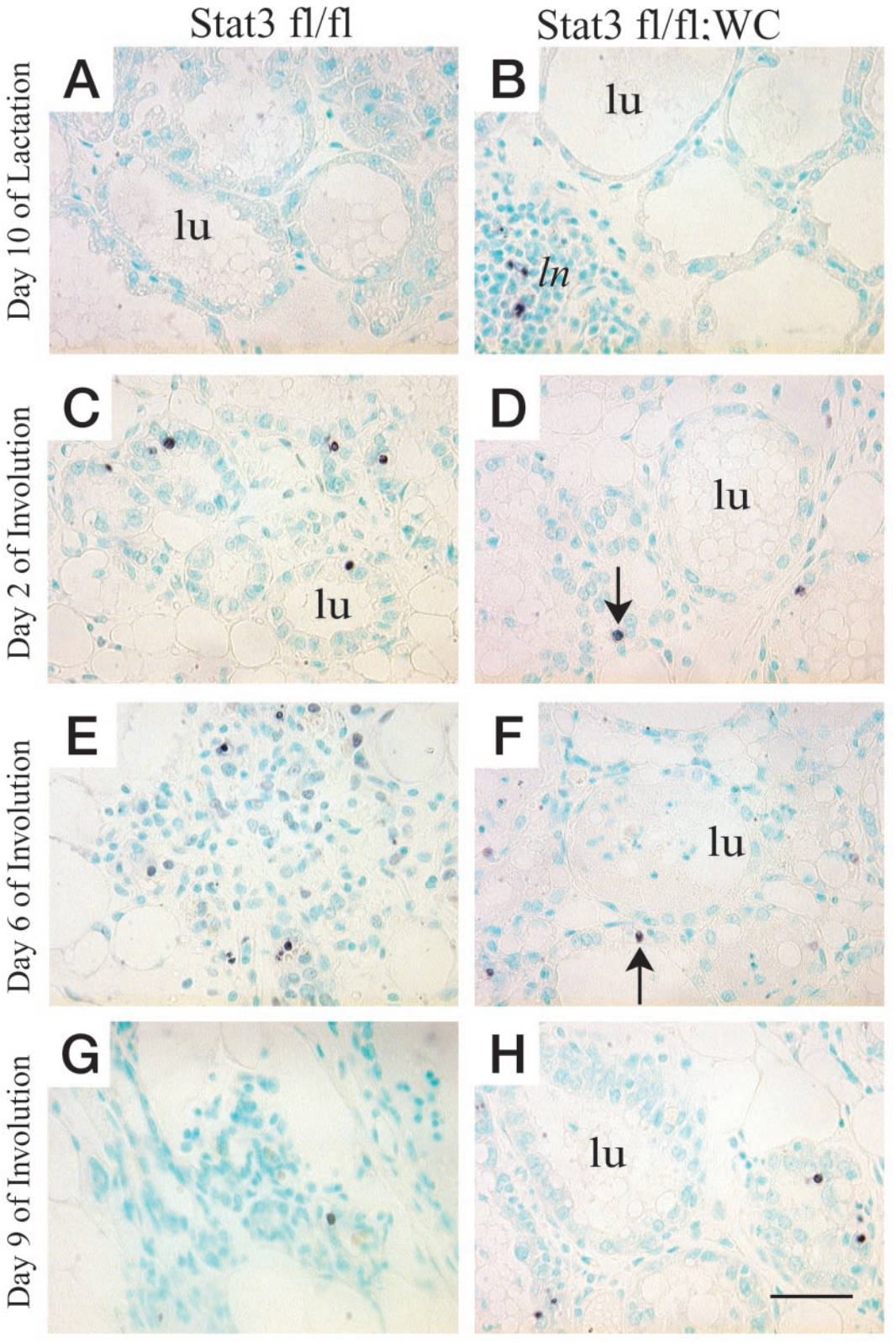

FIG. 5. Apoptosis in Stat3 fl/fl and Stat3 fl/fl;WC tissues. TUNEL staining was performed at d 10 of lactation and d 2, 6, and 9 of involution. Staining (A, B) at d 10 of lactation with no significant difference in apoptosis between the Stat3 fl/fl and Stat3 fl/fl;WC tissue. C, E, and G, d 2, 6, and 9 of involution from Stat3 fl/fl mammary tissue. D, F, and H, d 2, 6, and 9 of involution from Stat3 fl/fl;WC mammary tissue. There are fewer apoptotic cells present in the Stat3 fl/fl;WC tissue at 2 d of involution ($P < 0.05$). No statistically significant difference in the number of apoptotic cells could be determined for the other time points within an appropriate confidence interval. Many of the lumina in the Stat3 fl/fl;WC tissue remain fully expanded despite the occasional TUNEL positive apoptotic cells in near proximity. *Solid bar* represents 100 μm. ln, Lymph node; lu, lumina.

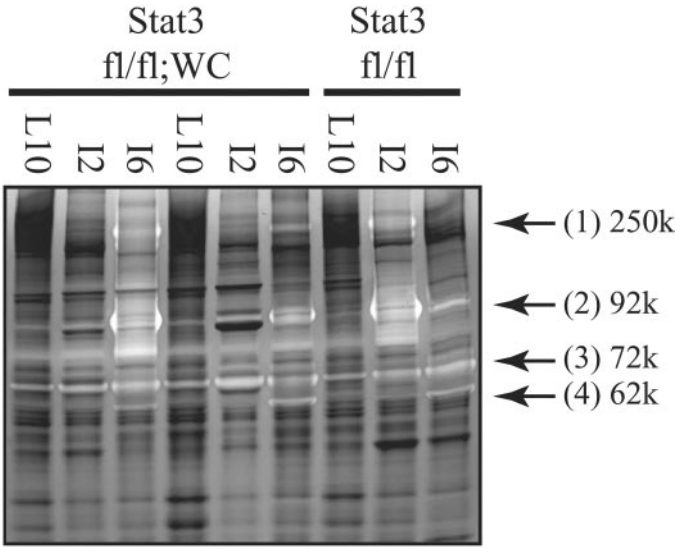

FIG. 6. Protease activity in Stat3 fl/fl and Stat3 fl/fl;WC tissues. Zymography was performed using 40 μg of protein from whole tissue lysate on unstained gelatin embedded Tris-glycine sodium dodecyl sulfate gels. The gels were subsequently Coomassie stained to show both the protein loading and relative protease activity. The image shown is captured in grayscale to clearly define the contrast of the bands within the gel that demonstrate both loading and protease activity (1). An unknown gelatinase at 250K (2). MMP9 activity (92k) is delayed until d 6 of involution in Stat3 fl/fl;WC mice, whereas the activity in the Stat3 fl/fl controls is visible at d 2 (3, 4). MMP2 activity of the proform and active at 72k and 62k, respectively. The zymogram assays were repeated in triplicate with different sample sets and the data shown is representative of glands surgically removed at 10 d of lactation and collected at the specified point during forced involution. Two sets of Stat3 fl/fl;WC tissue are shown to illustrate the slight variance in expression of zymogen activity at d 6 of involution.

T cells (13). Alternatively, it is possible that the Cre transgenes used in the two studies account for some of the differences.

Programed cell death is induced in the mammary gland within 12 h of the end of suckling (20) and increases sharply during the first phase of involution. Alterations in the levels of programmed cell death have been demonstrated in other transgenic and knockout mouse models that elicit a disruption of involution (12, 21–24). We were able to report a significant decrease in the level of apoptosis at d 2. However, there was not a complete absence of programmed cell death at either d 2 or 6 of involution, implying that the regulation of apoptosis is not entirely dependent on the presence of Stat3. We were able to show an increase in the level and duration of SGP-2 expression, although it is a protein of unknown function with respect to direct biological significance during mammary development or involution. Many possible mechanisms and roles have been suggested for SGP-2; however, the expression of SGP-2 is not necessary for the apoptotic process to proceed (25). SGP-2 is a secreted protein that has been shown to adhere to cells near the point of secretion and may infer a cytoprotection against certain apoptotic stimuli (26–28). Cell survival of mammary epithelium at the interface of lactation and involution has been shown to depend on the presence of the Bcl-x protein (16). Because the Bcl-x levels were unaltered in the absence of Stat3, cell survival must depend on other proteins.

The second stage of involution is characterized by the destruction of the lobuloalveolar unit and its surrounding extracellular matrix followed by a remodeling of the epithelial and stromal components (1, 29–31). MMPs are possible mediators of this process (32). A critical role for the MMPs and their inhibitors, tissue inhibitors of metalloproteinases, has been demonstrated in the development and maintenance of the mammary tissue (33, 34) and in the progression of involution (17, 22, 33). This function agrees with the disrupted protease pattern observed in the absence of Stat3. Therefore, we suggest that the loss of Stat3 causes a delay in the activity of the proteases and thereby alters the initiation and progression of the second irreversible stage during mammary involution. Stat3 and the IL6 family of cytokines that activate Stat3 can regulate the expression of MMP-3 and tissue inhibitor of metalloproteinase-1 (35, 36) *in vitro* and *in vivo*. However, the mechanism may be of indirect nature. Based on this and other studies (12), it is clear that functional loss of mammary epithelium after weaning requires the presence of Stat3, and we now show that Stat3 is also required for the activation of proteases *in vivo*. At this point, the molecular and mechanistic link between Stat3 and protease-mediated remodeling is not known.

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